FEBS Letters 589 (2015) 2441–2447

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Entropic clocks in the service of electrical signaling: 'Ball and chain' mechanisms for ion channel inactivation and clustering

Nitzan Zandany [1], Limor Lewin [1], Valerie Nirenberg [1], Irit Orr [1], Ofer Yifrach *

Department of Life Sciences and the Zlotowski Center for Neurosciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer Sheva 84105, Israel

ARTICLE INFO

Article history:
Received 30 April 2015
Revised 5 June 2015
Accepted 8 June 2015
Available online 22 June 2015

Edited by Wilhelm Just

Keywords:
'Ball and chain'
Clustering
Entropic chain
Inactivation
Intrinsic disorder
Scaffold protein
PSD-95
Voltage-dependent potassium channel

ABSTRACT

Electrical signaling in the nervous system relies on action potential generation, propagation and transmission. Such processes are dynamic in nature and rely on precisely timed events associated with voltage-dependent ion channel conformational transitions between their primary open, closed and inactivated states and clustering at unique membrane sites. In voltage-dependent potassium (Kv) channels, fast inactivation and clustering processes rely on entropic clock chains as described by 'ball and chain' mechanisms, suggesting important roles for such chains in electrical signaling. Here, we consider evidence supporting the proposed 'ball and chain' mechanisms for Kv channel fast inactivation and clustering associated with intrinsically disordered *N*- and *C*-terminal regions of the protein, respectively. Based on this comparison, we delineate the requirements that argue for such a process and establish the thermodynamic signature of a 'ball and chain' mechanism. Finally, we demonstrate how 'chain'-level alternative splicing of the Kv channel gene modulates the entropic clock-based 'ball and chain' inactivation and clustering channel functions underlying changes in electrical signaling. As such, the Kv channel model system exemplifies how linkage between alternative splicing and intrinsic disorder enables functional diversity.

1. Introduction

Our experience with protein structure–function teaches us that perturbations are often disruptive or destabilizing [1]. Scanning mutagenesis, sequence deletions or insertions, and the swapping of domains between homologous proteins used to create hybrid proteins all usually lead to a loss of function phenotype. Specifically, the mutant protein may be less stable, present slower catalytic activity, offer weaker affinity in interactions with ligands, DNA or other proteins, or shift protein conformational equilibrium towards the less optimally-packed state, just to name a few processes [1]. These effects of mutation reflect the fact that protein structures are delicate and have evolved over the course of million of years to intimately recognize their partner small molecule or protein/DNA substrates [1]. The realization that gain of function effects usually requires computational rational protein design, based on high-resolution structural information [2–4] further testifies to the structure–function narrative of molecular biochemistry.

With such notions in mind, it is easy to see how the findings of Aldrich and colleagues that long deletions in the cytoplasmic *N*-terminal inactivation domain of the prototypical *Shaker* voltage-dependent potassium (Kv) channel dramatically accelerated the entry of the channel into the inactivated state [5,6], were initially considered surprising (with inactivation referring to the blockage of K^+ currents through the channel, despite the continued presence of the depolarizing stimulus that leads to channel opening [7,8]). The authors demonstrated that the length of the *N*-terminal tail affected the kinetics of channel entry into the inactivated state in a systematic manner [5] and that a mutant channel lacking the entire *N*-terminal tail can still inactivate upon application of a short peptide corresponding to the first 20 *N*-terminal residues [6]. Aldrich and co-workers intuitively reasoned that such effects could be rationalized if the Kv channel *N*-terminal segment functioned as an extended flexible chain lacking any structure and thus proposed the 'ball and chain' mechanism to explain fast (*N*-type) channel inactivation (Fig. 1a) [5,6]. According to this mechanism, the first 20 *N*-terminal residues of the channel comprise an extended inactivation recognition motif (the 'ball') that is followed by a long stretch of amino acids (the 'chain'). The length of the extended chain sequence provides the degrees of freedom needed to seek and then bind the receptor site of the 'ball' at the inner cavity of the opened pore domain, thereby timing potassium

* Corresponding author. Fax: +972 8 647 9173.
E-mail address: ofery@bgu.ac.il (O. Yifrach).
[1] Fax: +972 8 647 9173.

http://dx.doi.org/10.1016/j.febslet.2015.06.010
0014-5793/

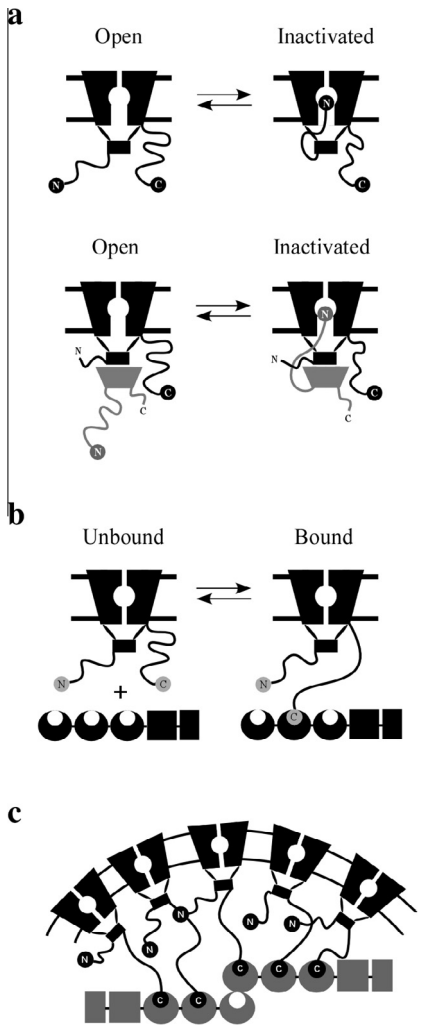

Fig. 1. 'Ball and chain' entropic clock-based mechanisms for ion channel fast inactivation and clustering. Schematic representations of the 'ball and chain' mechanisms for channel fast inactivation (a) and clustering (b). In the fast inactivation mechanism (a), the open (O) Kv channel pore inactivates (I) in a precisely timed manner, as determined by *N*-terminal chain length, upon binding of the 'chain'-tethered 'ball' to a receptor site within the inner cavity of the open pore. Such a mechanism can be intra-molecular or inter-molecular, depending on whether the 'ball and chain' sequence is attached to the channel itself (upper panel) or to the auxiliary β subunit (lower panel). In the clustering inter-molecular 'ball and chain' mechanism (b), Kv channel interaction with the membrane-associated PSD-95 scaffold protein is precisely timed, as determined by *C*-terminal chain length, upon binding of the 'chain'-tethered peptide 'ball' to PSD-95 PDZ domain(s). The membrane-embedded portion corresponds to the channel voltage-sensor and pore domains, while the rectangular shape corresponds to the T1 domain. The gray bout-shaped form in Fig. 1a corresponds to the auxiliary β subunit of the Kv channel α subunit. The crescent, box and rectangular shapes represent the PDZ, SH3 and guanylate kinase-like domains of the PSD-95 protein, respectively. (c) PSD-95-mediated Kv channel clustering at unique sites may be achieved by the multiple 'ball and chain' interactions, dictated by the stoichiometry of the interaction and the ability of PSD-95 to multimerize. Panels a and b were reproduced from Ref. [22] with permission.

current blockage through the ion conduction pathway [5,9–14]. Such a 'ball and chain' mechanism can be intra-molecular or inter-molecular, depending on whether the 'ball and chain' sequence is attached to the channel itself (upper panel in Fig. 1a) or to the auxiliary β subunit with which it interacts (lower panel). This 'ball and chain' model for Kv channel inactivation was suggested to be analogous to that used to describe voltage-dependent sodium channel inactivation by Armstrong and Bezanilla [15]. Furthermore, inactivation in mammalian Kv channels [16] and in Ca^{2+}-activated K^+ channels [17] was suggested to occur according to the same mechanism. More than a decade later, in the first reviews to systematically address the different functional roles played by intrinsically disordered protein segments, Dunker and colleagues rephrased the 'ball and chain' inactivation mechanism according to current terminology [18,19]. The Kv channel *N*-terminal tail was suggested to belong to the entropic chains class of intrinsically disordered protein segments and was assigned the function of timing channel fast inactivation, with chain length providing the determinant of time [18,19]. The Kv channel 'ball and chain' *N*-terminal tail sequence was thus proposed to function as entropic clock modulating fast channel inactivation [5,18,19].

Five years later, evidence began to accumulate demonstrating that the *Shaker* Kv channel protein makes use of a second entropic clock chain for binding to membrane-associated PSD-95 synaptic scaffold proteins [20–22] as may be described by analogous 'ball and chain' mechanism (Fig. 1b). This interaction underlies the clustering of many Kv channel molecules at unique membrane sites [23–26], a process important for efficient electrical signaling [25,27,28]. Here, however, the interaction involves the cytoplasmic *C*-terminal tail of the channel [22–24]. Similar to its *N*-terminal-based counterpart, the *C*-type 'ball and chain' mechanism relies on a channel tail that is comprised of an extended 'chain' bearing a conserved PDZ-recognition motif at its tip (the 'ball'), usually 4–8 residues in length, able to bind PDZ domains of PSD-95 scaffold protein partners (Fig. 1b) [22]. Deletion of only the terminal PDZ-binding motif eliminated PSD-95 binding and consequently, Kv channel clustering [21,23,26]. Furthermore, here too, the length of the Kv channel *C*-terminal tail determines affinity for the PSD-95 protein [21,22], as brought about by changes in association kinetics alone [22]. The Kv channel *C* terminal tail thus serves to time Kv channel–PSD-95 complex formation. How this 'ball and chain' interaction leads to channel clustering is determined by the stoichiometry of the interaction, dictated by the three PDZ interaction modules of PSD-95 and the four *C*-terminal tails of the oligomeric Kv channel, and by the ability of PSD-95 to multimerize [29–31] (Fig. 1c). This description of channel clustering hints for a possible functional linkage between the timing property of the Kv channel tail and the dynamics of channel cluster formation.

The Kv channel protein thus presents an interesting case whereby two completely distinct functions, namely channel fast inactivation and clustering, can be described by a 'ball and chain' mechanism associated with its intrinsically disordered cytoplasmic tails. Comparison of the similarities and differences of these two 'ball and chain' mechanisms should enhance our understanding of the function of intrinsically disordered protein segments as entropic clocks and their unique roles in electrical signaling. Furthermore, the analogy provides a mechanistic framework for studying Kv channel clustering, of which little is known, in a manner analogous to that employed for the study of Kv channel fast inactivation.

2. Entropic clocks and electrical signaling

Electrical signaling in the nervous system relies on the generation, propagation and transmission of action potentials, transient fluctuations in membrane voltage that spread along a neuron's axon and across the synapse [7]. Such fluctuations result from changes in membrane conductance, primarily to sodium and potassium ions, brought about by choreographed conformational gating transitions of voltage-activated sodium and potassium channels between their primary closed, open and inactivated states [7]. In the case of the modular Kv channel protein addressed here, the generation of action potentials primarily relies on the tight electro mechanical coupling between the membrane-spanning voltage sensor and the ion conduction pore

domain, an interaction responsible for channel opening (activation gating) [7,32,33]. Action potential shape and transmission along the axon and across the synapse are, however, modulated by the long cytoplasmic *N*- and *C*-terminal channel tails, involved in channel fast inactivation gating and clustering [5,6,22–25], respectively, as described by the 'ball and chain' mechanism. In the case of channel inactivation, the kinetics of K^+ current blockage through the channel upon 'ball' binding to its receptor site affects action potential shape, signal propagation and firing frequency [34–36]. *C*-terminal tail-based binding of the channel to PSD-95, on the other hand, leads to aggregation of many channel molecules at the post-synaptic density site [24,25] and potentially near nodes of Ranvier [37,38], thereby affecting membrane K^+ current density at these sites, traits important for action potential generation, propagation and transmission [27,28,39–41]. Thus, the two Kv channel entropic chains operating according to a 'ball and chain' mechanism to modulate channel inactivation and clustering point to important role(s) for entropic clocks in electrical signaling.

3. Criteria arguing for a 'ball and chain' mechanism

A 'ball and chain' binding mechanism involves three components, the two 'ball' and 'chain' sequence modules and a third structural module that provides the acceptor site for the 'ball'. Realizing whether a binding process may be described by entropic clock-based 'ball and chain' mechanism relies on the outcomes of perturbations at any of these components [5,9–13,22]. For such a mechanism to be valid, three criteria must be met. One needs to show (1) that the suspected 'ball and chain' sequence is indeed an intrinsically disordered random chain, (2) that the binding process is entropy-controlled and (3) that the 'chain' length times the association between the two binding partners/modules with no effect on complex dissociation. While the first two requirements serve to prove that the tail sequence under study is an entropic chain, the third requirement addresses what type of entropic chain is the tail. It should be noted that these requirements are 'chain'-centric [5,22], as they relate to modifications in tail length only, the outcome of which may be predicted by polymer chain theory [14,42]. A 'ball and chain' description, however, further carries predictions regarding the outcome of perturbations introduced into the 'ball' recognition motif, or at the receptor site for the 'ball'. Such perturbations are expected to mainly affect the dissociation kinetics of the 'ball' from its receptor site, rather than the association kinetics. As such, 'ball'-centric or 'chain'-centric criteria are complimentary. These orthogonal effects on the kinetics of protein/modules complex formation and dissociation upon 'ball' and/or 'chain' perturbations is the hallmark of a 'ball and chain' binding mechanism ([22], and see further below).

4. Experimental evidence for a 'ball and chain' mechanism for Kv channel fast inactivation and PSD-95 binding

The first requirement for a 'ball and chain' mechanism is for the tail to possess a random chain character. In the case of the Kv channel–PSD-95 interaction, a reductionist approach was taken in studying the isolated *C*-terminal tail of the archetypical *Shaker* B Kv channel protein removed of its channel and membrane contexts [21]. This tail sequence was recognized as being intrinsically disordered by all disorder-predicting algorithms [20,21]. Hydrodynamic and spectroscopic approaches subsequently verified this prediction [21]. Briefly, size exclusion chromatography revealed the tail protein to migrate unusually fast, as compared to standard molecular weight markers. Analytical ultra-centrifugation revealed that this anomaly was not a result of tail oligomerization. The tail protein thus assumes an extended non-globular shape. The far-UV CD spectrum of the tail further exhibited a random coil signature and its ^{1}H NMR spectrum was found to be typical of intrinsically disordered proteins, lacking the chemical shift dispersion typical of folded proteins possessing secondary and tertiary structures. Together, these results confirmed that the Kv channel *C*-terminal tail is an intrinsically disordered random chain [21]. In contrast to the reductionist approach used in the *C*-terminal tail experiments, the original studies on the *N*-terminal channel inactivation sequence [5,6] were conducted using the full-length Kv channel expressed in the plasma membrane of *Xenopus laevis* cells, thus hampering *N*-tail structural (or rather un-structural) characterization. In this respect, the extended nature of the *N*-terminal chain, as realized in the original studies that proposed the 'ball and chain' mechanism [5,6], was deduced based on functional studies alone (see below). NMR studies of isolated *N*-terminal channel 'ball' sequences alone revealed the existence of structural elements [43,44], although the significance of this observation was not clear in light of functional inactivation data [11,13]. Indeed, the *N*-terminal channel tail is predicted to be intrinsically disordered, as verified in our lab using the same reductionist approach as described above for the *C*-terminal tail (unpublished results).

The second criterion that must be met in validating a 'ball and chain' mechanism requires that the binding reaction be entropy-controlled, as dictated by chain length. This requirement intuitively follows consideration of the role of entropy contribution of binding reactions involving terminal extended chains. Shorter chains lose less entropy upon binding than do longer chains since the conformational entropy of the latter is restricted to a greater extent upon binding ([10,14,45], and see further below). In 'ball and chain' interaction systems, entropy thus unfavorably contributes to the binding energy. Pull-down assays, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) binding analyses all showed this to hold true for the *C*-terminal-based Kv channel–PSD-95 interaction [22]. Kv channel *C*-terminal tails of different lengths, generated upon systematic truncation of the wild type chain, yet all bearing identical PDZ-binding motifs, were tested for their binding to PSD-95 PDZ domains. Pull-down analysis qualitatively revealed that shorter, solid support-attached Kv channel tails captured more PDZ protein fragment than did longer chains. Quantitative analysis by SPR recapitulated the tail length-dependent nature of the interaction and revealed linear correlation between the binding energy of both proteins and Kv channel *C*-terminal tail length (Fig. 2a) [22]. The shorter the chain, the higher was the observed affinity for the partner protein. ITC analysis directly revealed the entropic modulation of the binding reaction. As can be seen in Fig. 2b describing the independence of the entropy and enthalpy contributions to Kv channel–PDZ interaction, the entropy but not the enthalpy of association was found to depend on Kv channel *C*-terminal tail length [22]. In fact, the entropic contribution is unfavorable for all chains, albeit less so for shorter chains. In summary, all three analyses considered here are compatible and argue for entropic modulation of the PSD-95–Kv channel interaction [22]. In the case of Kv channel fast inactivation, the involvement of entropy-controlled modulation of the inactivation process was recognized based on *N*-terminal chain length modulation of channel entry into the inactivated state [5,6] as well as on studies involving Kv channel inactivation using inactivation peptides [10]. Thus, both the *N*- and *C*-terminal tails are entropic chains, the lengths of which determine affinity for the 'ball' receptor site.

The third requirement that must be met in arguing for a 'ball and chain' mechanism addresses the type of entropic chain to which the tail corresponds, be it a linker, a spring, a bristle or a clock [18]. An entropic clock function requires that chain length primarily affects the kinetics of the binding phase and not the kinetics of unbinding (dissociation) [18,19,22]. Once 'ball' binding

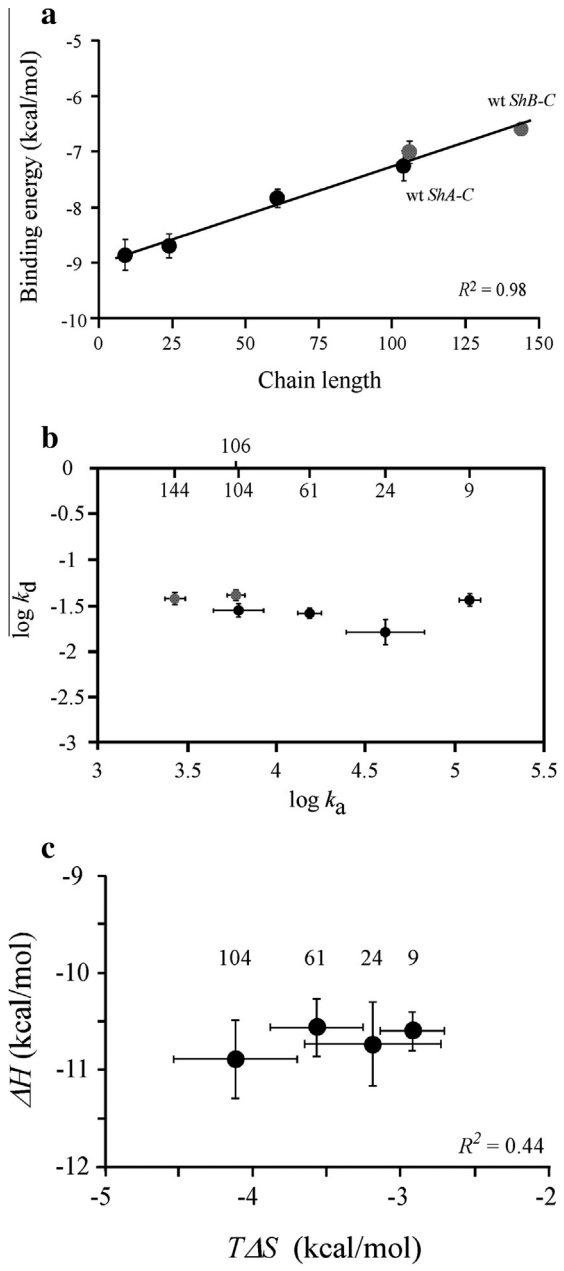

Fig. 2. *C*-terminal chain length modulation of the Kv channel–PSD-95 interaction. (a) The association binding energy ($-RT\ln K_A$) between the Kv channel and the PSD-95 protein linearly depends on *C*-terminal chain length, as achieved upon systematic 'chain' deletions. (b) Association–dissociation rate constant correlation plot of the Kv channel–PSD-95 interaction reveals the invariance of the two rate constants as a function of *C*-terminal chain length. In panels (a) and (b) the short and long natural Kv channel tail variants (designated *ShA-C* and *ShB-C*, respectively) are indicated by grey symbols. Numbers on the upper horizontal axis indicate tail 'chain' length. (c) Enthalpy–entropy correlation plot of the Kv channel–PSD-95 interaction reveals the invariance of the two quantities as a function of *C*-terminal 'chain' length. All figure panels were reproduced from Ref. [22] with permission.

to its receptor site has occurred, its dissociation is expected to take place at a similar rate, regardless of chain length. This outcome is expected if one considers that all 'chain' length variants of a protein bear the same 'ball' recognition motif and thus present similar surfaces for interaction of the 'ball' with its receptor site, whether within the same protein molecule or in a partner protein [10,14,22]. This was shown to be the case for both *N*- and *C*-terminal entropic chains. As reflected in Fig. 2c, describing the independence of the association and dissociation rate constants of the Kv channel–PSD-95 interaction, only the association rate constant (k_a) was found to depend upon 'chain' length [22]. Whereas k_a values for the longer and shorter channel 'chains' varied by almost three orders of magnitude, protein dissociation was independent of chain length. Furthermore, a plot of the ratio of association rate constants for mutant and wild type channel tails as a function of 'chain' length difference (ΔN) revealed a power law dependence expected for a random flight 'chain' seeking its 'ball'-binding partner [22,14]. As expected, a complimentary set of experiments involving perturbations introduced at terminal 'ball' PDZ-binding motif peptides, all producing variants with an identical length, revealed effects solely on the dissociation rate constant from PSD-95 PDZ domains, without any impact on the association kinetics [46]. Furthermore, similar behavior was observed when the PDZ domains themselves (i.e., the receptor sites for the 'ball') were mutated [46]. Taken together, these observations clearly demonstrate the timing property of the Kv channel–PSD-95 association as a modulated by *C*-terminal 'chain' length. The results described mirror earlier observations made for the *N*-terminal segment with respect to channel inactivation [5,6,10]. First, *N*-terminal tail length affected the kinetics of channel entry into the inactivated state [5,6]. Second, power law-dependence with a value of 3/2 typical for a random flight 'ball and chain' mechanism was also observed for the relation between normalized entry into inactivation rate constants and Kv channel *N*-terminal chain length [14]. Third, studies of *Shaker* K^+ channel block by inactivation peptides revealed that mutations introduced within the first half of the inactivation peptide 'ball', spanned by mostly hydrophobic amino acids and which comprise the direct recognition inactivation motif, were found to affect only the complex dissociation rate constant, with no effect on channel-peptide association rate constants [10]. Finally, mutation or perturbations in the inactivation 'ball' receptor site, particularly of residues facing the central ion conduction pathway at the inner channel vestibule, were further found to affect the recovery from inactivation rate constant, with no effects on entry into inactivation kinetics [13]. To summarize, the data surveyed here demonstrate how the three criteria that argue for the use of a 'ball and chain' entropic clock have been fulfilled for both Kv channel fast inactivation and PSD-95-mediated clustering, implying that the mechanisms underlying these distinct processes are, in fact, analogous.

5. Alternative splicing in Kv channels modulates 'ball and chain' interactions

The 'ball and chain' mechanisms of Kv channel fast inactivation and PSD-95-mediated clustering were postulated based on deletion-shortened *N*- or *C*-chains [5,22]. This raises the question of the physiological/cellular relevance of such a mechanism. The clear-cut answer to this question is provided when considering alternative RNA splicing of the prototypical *Shaker* Kv channel gene. Such processing was shown to occur only at message segments that encode for either the *N*- or *C*-terminal tails, thus leading to the appearance of terminal chains of different lengths [47,48]. The fact that spatial–temporal differences exist in the expression patterns of the *N*- or *C*-terminal spliced variant chains during development or in different tissues [47–50] further points to the physiological importance of alternative splicing in regulating channel inactivation or clustering, as governed by the 'ball and chain' mechanism. Indeed, natural *N*-terminal chain splice variants were shown to exhibit distinct kinetics of channel entry into the inactivated state [51,52]. Furthermore, the two short and long Kv channel *C*-terminal chain splice variants (designated *A* and *B*, respectively) were shown to exhibit distinct affinities for PSD-95, distinct PSD-95-mediated channel cell surface expression levels and distinct clustering patterns [22]. The short tail *A* variant

presented higher affinity towards PSD-95 and higher cell surface expression level than did the long tail *B* variant. It further supported larger PSD-95-mediated channel clusters in Schneider cell membranes as reflected in Fig. 3. Thus, molecular distinctions reflected in the differential interactions of the tail variants with PSD-95 translate into functional differences in the context of cellular channel clustering [22]. Splicing-based changes in potassium current density at unique membrane sites may subsequently lead to changes in action potential propagation and transmission [39–41]. Taken together, the *Shaker* Kv channel model protein system, with its functional *N*- and *C*-type 'ball and chain' inactivation and clustering mechanisms, exemplifies how linkage between alternative splicing and intrinsic disorder enables functional diversity to modulate 'ball and chain' interactions [53,54], in this particular case, in the context of neuronal electrical excitability.

6. A thermodynamic signature of a 'ball and chain' mechanism

The 'ball and chain' binding mechanism analyzed here in detail for Kv channel fast inactivation and clustering and its regulation by alternative splicing at the 'chain' level provide a general lesson on the role of intrinsically disordered entropic chains in modulating binding processes. The 'ball and chain' mechanism represents one of the most clear-cut cases where the entropy and enthalpy contributions of a binding reaction can be exclusively attributed to distinct sequence modules. Adopting a 'chain'-centric point of view, whereas the 'chain' module primarily controls the entropy of the binding reaction, as manifested by the dependence ΔS on chain length [22], the 'chain'-tethered 'ball' module primarily determines the enthalpy of the binding reaction, as manifested by the invariance of ΔH as a function of chain length. These 'ball' and 'chain' sequence modules further determine the association and dissociation kinetics of the two proteins. Whereas chain length modulates association kinetics, the dissociation rate constant is determined by the 'ball' recognition motif (and by its receptor docking site). We thus propose that the $\log k_d - \log k_a$ and $\Delta H - \Delta S$ profiles, in which the measured parameters show no dependence on each other upon changes in chain length (Fig. 2b and c, respectively), correspond to a thermodynamic signature of entropy-controlled 'ball and chain' binding mechanisms involving entropic clocks. Though nature uses a 'chain'-centric approach in regulating the 'ball and chain' clustering interactions, as manifested by 'chain'-level alternative splicing of the *C* terminal tail [47,48], both 'chain'- and 'ball'-centric approaches are taken for the *N* terminal segment. In any case, adopting a 'ball'-centric perturbation approach would also reveal invariance of the $\log k_d - \log k_a$ and $\Delta H - \Delta S$ parameters. However, these effects would be orthogonal to those observed in the case where 'chain' length is varied, i.e., 'ball' mutations are expected to affect ΔH and k_d alone without changing ΔS or k_a. No matter whether a 'ball'- or 'chain'-centric approach is adopted, the nature of this signature contrasts with the linear correlation between ΔH and ΔS often observed in structure–function studies describing protein–protein interactions involving two globular proteins, a phenomenon referred to as enthalpy–entropy compensation [55].

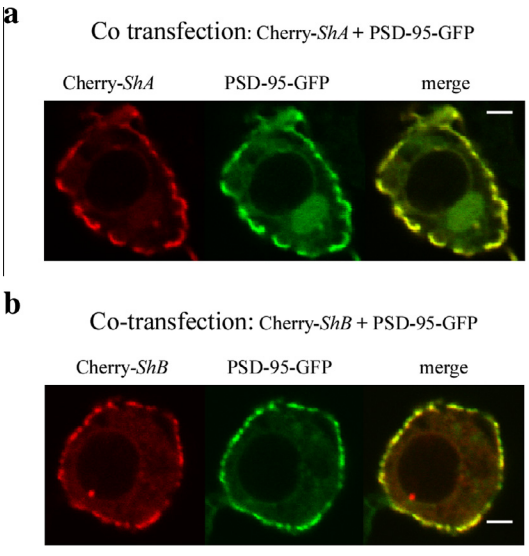

Fig. 3. Alternative splicing-based modulation of Kv channel clustering. The *Shaker A* and *B* chain splice variants exhibit differences in cluster area size. (a and b) Confocal microscopy analysis of *Drosophila* S2 Schneider cells co-expressing PSD-95-GFP and either the mCherry-ShA short chain variant (a) or mCherry-ShB long chain variant (b). For each representative cell, three images are shown, with the red channel-associated and green PSD-95-associated fluorescence signals presented in the left and middle columns, respectively. The merged image of each cell is shown in the right column. Scale bars correspond to 2 μm (reproduced from Ref. [22] with permission).

7. Inactivation and clustering 'ball and chain' mechanisms: differences and variations

As outlined in this contribution, both Kv channel inactivation and clustering are based on entropic chains with timing function, as can be described by a simple two state (bound and unbound) 'ball and chain' mechanism. While this reductionist description provides a reference framework to analyze, compare and interpret data, differences between the inactivation and PSD-95 binding functions of the Kv channel exist, as well as variations on the 'ball and chain' mechanism, as outlined below.

One principal difference between the two 'ball and chain' mechanisms is related to the location of the receptor site for the 'ball', being either on the same molecule (for inactivation) or on a different molecule (for clustering) (Fig. 1). Thus, the molecularity of the binding reaction is different in each case, being first order for the inactivation process and second-order for Kv channel–PSD-95 interaction, a fact that influences the effective concentration of the 'ball' near its receptor site [5,14]. This, at first, suggests that the 'fly casting' metaphor [56,57] better describes the Kv channel–PSD-95 interaction. After all, the membrane-embedded Kv channel uses its extended *C*-terminal tail, with its 'hook' recognition motif, to 'cast' for its cytoplasmic PSD-95 scaffold protein partner. Still, we choose to adhere to the 'ball and chain' metaphor primarily because of the historical context [5,6,15] and in light of compatible lines of evidence considered above. Moreover, inherent to the 'fly casting' mechanism is the 'folding upon binding' scenario (even if limited or local). While restriction of the (Kv channel) 'chain' conformational ensemble upon PSD-95 binding definitely occurs, the chain remains extended, with no folding upon binding or 'disorder to order' transition occurring, as supported by both limited proteolysis analysis [21] and phosphorylation site accessibility assays (unpublished results). In any case, the analogy between the *N*- and *C*-terminal-based 'ball and chain' mechanisms is further strengthened if one considers that PSD-95 is *a priori* membrane-associated (due to its palmitoylation [58] and potential interactions with other membrane protein partners (e.g., NMDA receptors [23])), as well as the common variation on Kv channel fast inactivation where the 'ball and chain' sequence is not carried by the channel itself but rather on the auxiliary β subunit of the channel with which it interacts [12,59] (Fig. 1a, bottom panel).

Finally, variations on the 'ball and chain' mechanism exist. First, transient states may occur along the binding pathway. For example, with respect to channel inactivation, the inactivation 'ball' contains a 10 amino acid-long stretch enriched in positive charges, adjacent

to the preceding inactivation recognition motif [5,6]. Mutations at these positions affected both entry into and recovery from inactivation [9,60]. It has been argued that this positive amino acid stretch is involved in long-range electrostatic interactions that may direct the chain towards the 'ball' receptor site at the inner cavity of the open pore [5,9,60]. This sequence was further suggested to be involved in a rate-determining pre-block state, thus separating the binding and blocking events [11]. Furthermore, other charged *N*-terminal chain residues were suggested to play a role in long-range electrostatics, as well as in forming intimate interactions with assembly (T1) domain residues on the outer part of the intracellular entryway windows [61]. Restraining of the inactivation chain by 'redox state'-dependent electrostatic interactions (thus inhibiting fast inactivation) was also reported for the Kvβ1 'ball and chain' sequence [62]. These observations suggested the 'chain' to be 'sticky' (i.e., active rather than passive), implying the existence of additional transient states in the fast inactivation pathway [61–63]. Still, even with these 'chain'-level variations, the chain assumed to be extended. Furthermore, 'ball'-level regulation of the binding process by direct recognition motif phosphorylation [41], oxidation [16], protonation [64] or heme-binding [65] has been documented. Finally, regulation at the 'ball' acceptor site-level is possible, as demonstrated by RNA editing of the message for a residue found at the Kv channel inner vestibule [13]. The take-home message here is that these variations may serve to regulate 'ball and chain' interactions yet do not undermine the principal reductionist-level mechanistic analogy between the inactivation and clustering functions of the Kv channel adopted here.

8. Concluding remarks

This contribution has focused on the entropic clock function of intrinsically disordered protein segments, as exemplified by the 'ball and chain' mechanisms associated with ion channel function. Such a mechanism was initially suggested for voltage-dependent sodium channel inactivation in the 1970's [15] and later for Kv channel fast inactivation in the 1990's [5,6], when disordered protein segments were mostly encountered by crystallographers, who considered them as bothersome protein segments that hampered protein crystallization. The concept of intrinsically disordered protein segments, with their diverse functional roles, was not yet consolidated and analysis methods to assess the 'un-structured' state of such segments were scarce at the time. In this respect, proposal of the 'ball and chain' fast inactivation mechanism based on functional data alone showed considerable foresight.

Much has changed since the seminal studies of Aldrich and co-workers. The many distinct roles intrinsically disordered protein segments serve in almost all cellular processes [18,66–72] support the 'no-structure–function' scenario that compliments the traditional structure–function narrative [67,70–72]. With this in mind, and considering the historic context of ion channel biophysics, we have recently shown that Kv channel clustering occurs according to a 'ball and chain' mechanism, analogous in essence to the original inactivation mechanism. These entropic clock-based mechanisms share a unique thermodynamic signature and are modulated by alternative splicing of the *Shaker* Kv channel gene to produce *N*- or *C*-terminal chain length variants, thus serving to affect the electrical properties of the channel underlying action potential shape, propagation and transmission.

Acknowledgements

This research was supported by the Israel Science Foundation (Grant 488/12 to O.Y.). The authors declare of no conflict of interests.

References

[1] Pakula, A.A. and Saur, R.T. (1989) Genetic analysis of protein stability and function. Annu. Rev. Genet. 23, 289–310.
[2] Kries, H., Blomberg, R. and Hilvert, D. (2013) De novo enzymes by computational design. Curr. Opin. Chem. Biol. 17, 221–228.
[3] Feldmeier, K. and Hocker, B. (2013) Computational protein design of ligand binding and catalysis. Curr. Opin. Chem. Biol. 17, 929–933.
[4] Damborsky, J. and Brezovsky, J. (2014) Computational tools for designing and engineering enzymes. Curr. Opin. Chem. Biol. 19, 8–16.
[5] Hoshi, T., Zagotta, W.N. and Aldrich, R.W. (1990) Biophysical and molecular mechanisms of Shaker potassium channel inactivation. Science 250, 533–538.
[6] Zagotta, W.N., Hoshi, T. and Aldrich, R.W. (1990) Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. Science 250, 568–571.
[7] Hille, B. (2001) Ion Channels of Excitable Membranes, Sinauer Associates Inc., Sunderland, MA.
[8] Rasmusson, R.L., Morales, M.J., Wang, S., Liu, S., Campbell, D.L., Brahmajothi, M.V. and Strauss, H.C. (1998) Inactivation of voltage-gated cardiac K+ channels. Circ. Res. 82, 739–750.
[9] Demo, S.D. and Yellen, G. (1991) The inactivation gate of the Shaker K+ channel behaves like an open-channel blocker. Neuron 7, 743–753.
[10] Murrell-Lagnado, R.D. and Aldrich, R.W. (1993) Interactions of amino terminal domains of Shaker K channels with a pore blocking site studied with synthetic peptides. J. Gen. Physiol. 102, 949–975.
[11] Zhou, M., Morais-Cabral, J.H., Mann, S. and MacKinnon, R. (2001) Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors. Nature 411, 657–661.
[12] Aldrich, R.W. (2001) Fifty years of inactivation. Nature 411, 643–644.
[13] Gonzalez, C., Lopez-Rodriguez, A., Srikumar, D., Rosenthal, J.J. and Holmgren, M. (2011) Editing of human K(V)1.1 channel mRNAs disrupts binding of the N-terminus tip at the intracellular cavity. Nat. Commun. 2, 436–441.
[14] Timpe, L.C. and Peller, L. (1995) A random flight chain model for the tether of the Shaker K+ channel inactivation domain. Biophys. J. 69, 2415–2418.
[15] Armstrong, C.M. and Bezanilla, F. (1977) Inactivation of the sodium channel. J. Gen. Physiol. 70, 567–590.
[16] Ruppersberg, J.P., Stocker, M., Pongs, O., Heinemann, S.H., Frank, R. and Koenen, M. (1991) Regulation of fast inactivation of cloned mammalian IK(A) channels by cysteine oxidation. Nature 352, 711–714.
[17] Solaro, C.R. and Lingle, C.J. (1992) Trypsin-sensitive, rapid inactivation of a calcium-activated potassium channel. Science 257, 1694–1698.
[18] Dunker, A.K. et al. (2001) Intrinsically disordered protein. J. Mol. Graph. Model. 19, 26–59.
[19] Dunker, A.K., Brown, C.J., Lawson, J.D., Iakoucheva, L.M. and Obradovic, Z. (2002) Intrinsic disorder and protein function. Biochemistry 41, 6573–6583.
[20] Magidovich, E., Fleishman, S.J. and Yifrach, O. (2006) Intrinsically disordered *C*-terminal segments of voltage-activated potassium channels: a possible fishing rod-like mechanism for channel binding to scaffold proteins. Bioinformatics 22, 1546–1550.
[21] Magidovich, E., Orr, I., Fass, D., Abdu, U. and Yifrach, O. (2007) Intrinsic disorder in the *C*-terminal domain of the Shaker voltage-activated K^+ channel modulates its interaction with scaffold proteins. Proc. Natl. Acad. Sci. USA 104, 13022–13027.
[22] Zandany, N., Marciano, S., Magidovich, E., Frimerman, T., Yehezkel, R., Shem-Ad, T., Lewin, L., Abdu, U., Orr, I. and Yifrach, O. (2015) Alternative splicing modulates Kv channel clustering through a molecular Ball and Chain mechanism. Nat. Commun. 6 (6488), 6500.
[23] Kim, E., Niethammer, M., Rothschild, A., Jan, Y.N. and Sheng, M. (1995) Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. Nature 378, 85–88.
[24] Tejedor, F.J. et al. (1997) Essential role for dlg in synaptic clustering of Shaker K channels in vivo. J. Neurosci. 17, 152–159.
[25] Ruiz-Canada, C., Koh, Y.H., Budnik, V. and Tejedor, F.J. (2002) DLG differentially localizes Shaker K+ channels in the central nervous system and retina of Drosophila. J. Neurochem. 82, 1490–1501.
[26] Tiffany, A.M. et al. (2000) PSD-95 and SAP-97 exhibit distinct mechanisms for regulating K+ channel surface expression and clustering. J. Cell Biol. 148, 147–158.
[27] Budnik, V., Zhong, Y. and Wu, C.-F. (1990) Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. J. Neurosci. 10, 3754–3768.
[28] Zhong, Y., Budnik, V. and Wu, C.-F. (1992) Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. J. Neurosci. 12, 644–651.
[29] Christopherson, K.S., Sweeney, N.T., Craven, S.E., Kang, R., El-Husseini, Ael.-D. and Bredt, D.S. (2003) Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks. J. Cell Sci. 116, 3213–3219.
[30] Hsueh, Y.P., Kim, E. and Sheng, M. (1997) Disulfide-linked head-to-head multimerization in the mechanism of ion channel clustering by PSD-95. Neuron 18, 803–814.
[31] Gomperts, S.N. (1996) Clustering membrane proteins: it's all coming together with the PSD-95/SAP90 protein family. Cell 84, 659–662.
[32] Lu, Z., Klem, A.M. and Ramu, Y. (2001) Ion conduction pore is conserved among potassium channels. Nature 413, 809–813.

[33] Long, S.B., Campbell, E.B. and Mackinnon, R. (2005) Voltage sensor of Kv1.2: structural basis of electromechanical coupling. Science 309, 903–908.
[34] Debanne, D., Guerineau, N.C., Gahwiler, B.H. and Thompson, S.M. (1997) Action-potential propagation gated by an axonal I(A)-like K+ conductance in hippocampus. Nature 389, 286–289.
[35] Giese, K.P. et al. (1998) Reduced K+ channel inactivation, spike broadening, and after-hyperpolarization in Kvbeta1.1-deficient mice with impaired learning. Learn. Mem. 5, 257–273.
[36] Johnston, D., Hoffman, D.A., Colbert, C.M. and Magee, J.C. (1999) Regulation of back-propagating action potentials in hippocampal neurons. Curr. Opin. Neurobiol. 9, 288–292.
[37] Ogawa, Y., Oses-Prieto, J., Kim, M.Y., Horresh, I., Peles, E., Burlingame, A.L., Trimmer, J.S., Meijer, D. and Rasband, M.N. (2010) ADAM22, a Kv1 channel-interacting protein, recruits membrane-associated guanylate kinases to juxtaparanodes of myelinated axons. J. Neurosci. 30, 1038–1048.
[38] Trimmer, J.S. (2015) Subcellular localization of K+ channels in mammalian brain neurons: remarkable precision in the midst of extraordinary complexity. Neuron 85, 238–256.
[39] Århem, P., Klement, G. and Blomberg, C. (2006) Channel density regulation of firing patterns in a cortical neuron model. Biophys. J. 90, 4392–4404.
[40] Zeberg, H., Blomberg, C. and Århem, P. (2010) Ion channel density regulates switches between regular and fast spiking in soma but not in axons. PLoS Comput. Biol. 6, e1000753.
[41] Zeberg, H., Robinson, H.P. and Arhem, P. (2015) Density of voltage-gated potassium channels is a bifurcation parameter in pyramidal neurons. J. Neurophysiol. 113, 537–549.
[42] Liebovitch, L.S., Selector, L.Y. and Kline, R.P. (1992) Statistical properties predicted by the ball and chain model of channel inactivation. Biophys. J. 63, 1579–1585.
[43] Antz, C., Geyer, M., Fakler, B., Schott, M.K., Guy, H.R., Frank, R., Ruppersberg, J.P. and Kalbitzer, H.R. (1997) NMR structure of inactivation gates from mammalian voltage-dependent potassium channels. Nature 385, 272–275.
[44] Antz, C., Bauer, T., Kalbacher, H., Frank, R., Covarrubias, M., Kalbitzer, H.R., Ruppersberg, J.P., Baukrowitz, T. and Fakler, B. (1999) Structure of the inactivating gate from the Shaker voltage gated K+ channel analyzed by NMR spectroscopy. Nat. Struct. Biol. 6, 146–150.
[45] Flock, T., Weatheritt, R.J., Latysheva, N. and Babu, M.M. (2014) Controlling entropy to tune the functions of intrinsically disordered regions. Curr. Opin. Struct. Biol. 26, 62–72.
[46] Haq et al. (2012) Side-chain interactions form late and cooperatively in the binding reaction between disordered peptides and PDZ domains. J. Am. Chem. Soc. 134, 599–605.
[47] Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Multiple potassium-channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. Nature 331, 137–142.
[48] Pongs, O. et al. (1988) Shaker encodes a family of putative potassium channel proteins in the nervous system of Drosophila. EMBO J. 7, 1087–1096.
[49] Mottes, J.R. and Iverson, L.E. (1995) Tissue-specific alternative splicing of hybrid Shaker/lacZ genes correlates with kinetic differences in Shaker K+ currents in vivo. Neuron 14, 613–623.
[50] Rogero, O., Hammerle, B. and Tejedor, F.J. (1997) Diverse expression and distribution of *Shäker* potassium channels during the development of the Drosophila nervous system. J. Neurosci. 17, 5108–5118.
[51] Timpe, L.C., Jan, Y.N. and Jan, L.Y. (1988) Four cDNA clones from the Shaker locus of *Drosophila* induce kinetically distinct A-type potassium currents in Xenopus oocytes. Neuron 1, 659–667.
[52] Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. and Rudy, B. (1988) A-type potassium channels expressed from Shaker locus cDNA. Proc. Natl. Acad. Sci. USA 85, 5723–5727.
[53] Romero, P.R. et al. (2006) Alternative splicing in concert with protein intrinsic disorder enables increased functional diversity in multicellular organisms. Proc. Natl. Acad. Sci. USA 103, 8390–8395.
[54] Buljan, M., Chalancon, G., Dunker, A.K., Bateman, A., Balaji, S., Fuxreiter, M. and Babu, M.M. (2013) Alternative splicing of intrinsically disordered regions and rewiring of protein interactions. Curr. Opin. Struct. Biol. 23, 443–450.
[55] Cooper, A. (1999) Thermodynamic analysis of biomolecular interactions. Curr. Opin. Chem. Biol. 3, 557–563.
[56] Shoemaker, B.A. et al. (2000) Speeding molecular recognition by using the folding funnel: the fly-casting mechanism. Proc. Natl. Acad. Sci. USA 97, 8868–8873.
[57] Trizac, E., Levy, Y. and Wolynes, P.G. (2010) Capillarity theory for the fly-casting mechanism. Proc. Natl. Acad. Sci. USA 107, 2746–2750.
[58] Topinka, J.R. and Bredt, D.S. (1998) N terminal palmitoylation of PSD-95 regulates association with cell membranes and interaction with K+ channel Kv 1.4. Neuron 20, 125–134.
[59] Rettig, J. et al. (1994) Inactivation properties of voltage-gated K+ channels altered by presence of beta-subunit. Nature 369, 289–294.
[60] Murrell-Lagnado, R.D. and Aldrich, R.W. (1993) Energetics of Shaker K channels block by inactivation peptides. J. Gen. Physiol. 102, 977–1003.
[61] Prince-Carter, A. and Pfaffinger, P.J. (2009) Multiple intermediate states precede pore block during N-type inactivation of a voltage-gated potassium channel. J. Gen. Physiol. 134, 15–34.
[62] Pan, Y., Weng, J., Levin, E.J. and Zhou, M. (2011) Oxidation of NADPH on Kvbeta1 inhibits ball- and-chain type inactivation by restraining the chain. Proc. Natl. Acad. Sci. USA 108, 5885–5890.
[63] Pfaffinger, P.J. (2013) A conserved pre-block interaction motif regulates potassium channel activation and N-type inactivation. PLoS One, e79891.
[64] Padanilam, B.J. et al. (2002) Molecular determinants of intracellular pH modulation of human Kv1.4 N-type inactivation. Mol. Pharmacol. 62, 127–134.
[65] Sahoo, N. et al. (2012) Heme impairs the ball-and-chain inactivation of potassium channels. Proc. Natl. Acad. Sci. USA 110, E4036–E4044.
[66] Uversky, V.N., Gillespie, J.R. and Fink, A.L. (2000) Why are "natively unfolded" proteins unstructured under physiologic conditions? Proteins 41, 415–427.
[67] Tompa, P. (2002) Intrinsically unstructured proteins. Trends Biochem. Sci. 27, 527–533.
[68] Wright, P.E. and Dyson, J.D. (2009) Linking folding and binding. Curr. Opin. Struct. Biol. 19 (31), 38.
[69] Gsponer, J. and Babu, M.M. (2009) The rules of disorder or why disorder rules. Prog. Biophys. Mol. Biol. 99, 94–103.
[70] Uversky, V.N. and Dunker, A.K. (2010) Understanding protein non-folding. Biochem. Biophys. Acta 1804, 1231–1264.
[71] van der Lee, R. et al. (2014) Classification of intrinsically disordered regions and proteins. Chem. Rev. 114, 6589–6631.
[72] Wright, P.E. and Dyson, H.J. (1999) Intrinsically unstructured proteins: re-assessing the protein structure–function paradigm. J. Mol. Biol. 293, 321–331.